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14. ABSTRACT
The threat of bioterrorism and the use of biological weapons against both military personnel and civilian populations has become an increasing concern for governments around the world. The 1984 Rajneeshee Salmonella attack, 2001 anthrax letter attacks, 2003 SARS outbreak, 2009 H1N1 swine flu pandemic, and the current US flu epidemic all illustrate our vulnerability to both deliberate and natural outbreaks of infectious disease and underscore the necessity of effective antimicrobial and antiviral therapeutics. The prevalence of antibiotic resistant strains and the ease by which antibiotic resistance can be engineered into bacteria further highlights the need for continued development of novel antibiotics against new bacterial targets. This research project directly addresses this need through the development of a broad spectrum inhibitor of the biothreat agents *Francisella tularensis* and *Yersinia pestis*.

During this period of performance, we have optimized assays with the *Y. pestis* MEP synthase and the *F. tularensis* MEP cytidylyltransferase for use in HTS. The screening of natural product and rationally designed libraries has identified a novel inhibitor that binds to an allosteric site on MEP synthase. We confirmed this allosteric activity with the MEP synthase homologs obtained from *F. tularensis* and *M. tuberculosis*. This allosteric site has not been previously identified and represents a new site for the rational design of a new chemical class of antimicrobial drugs targeting MEP synthase. Additionally, our screening has highlighted a rationally designed bisubstrate inhibitor of MEP synthase that behaves as a tightly bound inhibitor, binding to the NADPH site and causing a conformation change that subsequently "locks" the inhibitor into the DXP site. And our initial screening has also identified an effective inhibitor of MEP cytidylyltransferase.

15. SUBJECT TERMS
Enzymology, MEP pathway, bacteria, isoprene, drug discovery, antibiotics, screening, biothreat, bisubstrate inhibitor, allosteric inhibitor

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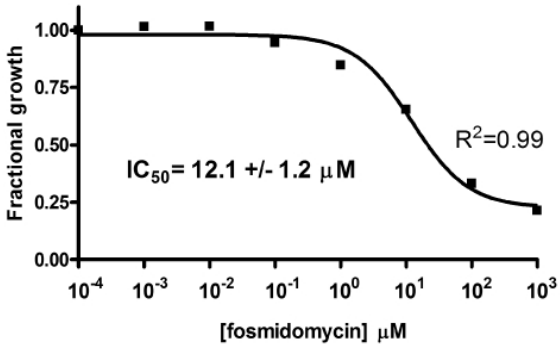
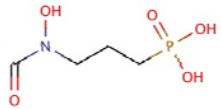
Introduction

The long term objective of this research is to identify and develop a broad spectrum inhibitor of *Francisella tularensis* and *Yersinia pestis*. The methylerythritol phosphate (MEP) biosynthetic pathway of *Francisella tularensis* and *Yersinia pestis* provide multiple enzymes that may be targeted for inhibitor development. This pathway is utilized by bacteria, apicomplexan protozoa, and plants for isoprenoid biosynthesis. Isoprenic compounds are vital for cellular processes such as electron transport, cell wall and membrane biosynthesis, and signal transduction. Despite their structural and functional diversity, all isoprenoids are derived from two building blocks, isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which originate from either the MEP pathway or the mevalonic acid (MVA) pathway depending on the organism. Humans acquire isoprenes through the nonhomologous MVA pathway, making enzymes in the MEP pathway very attractive targets for antimicrobial development.

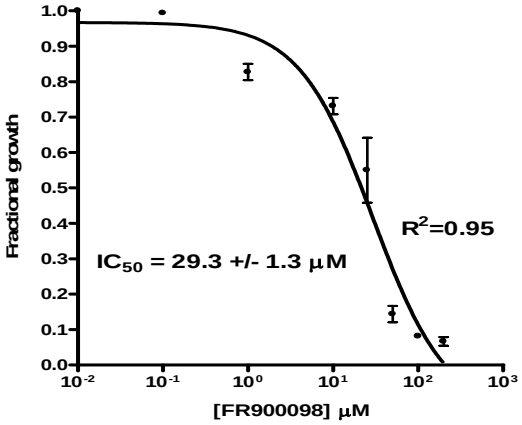
Body

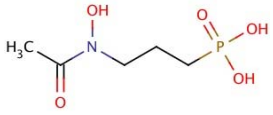
We hypothesize that inhibitors of the MEP pathway in *Francisella tularensis* and *Yersinia pestis* will serve as effective antibiotics by blocking isoprene biosynthesis. In strong support of this hypothesis, we have demonstrated the dose-dependent inhibition of *F. tularensis* and *Y. pestis* growth *in vitro* using the compounds fosmidomycin and FR900098 (Figure 1).





Fosmidomycin





FR900098

Figure 1. Growth inhibition of *F. tularensis* (top) and *Y. pestis* (bottom) by the compounds fosmidomycin and FR900098 (these molecules inhibit MEP synthase, an enzyme in the MEP pathway). The structures of the compounds are shown.

To test this hypothesis, the Couch lab at George Mason University is collaborating with Walter Reed Army Institute of Research (WRAIR) in the screening of compound diversity libraries using enzyme-based assays for lead inhibitor discovery, evaluation of lead inhibitors in microbial growth assays, determining X-ray crystal structures of the MEP pathway enzymes MEP synthase and MEP cytidylyltransferase in complex with inhibitors, and using this information to design and synthesize novel broad spectrum antibacterials. Five specific aims are being pursued in the Couch lab:

Aim 1) Express, purify, and characterize recombinant *Y. pestis* MEP synthase and MEP cytidylyltransferase.

We have successfully cloned, expressed, purified, and characterized the *Y. pestis* CO92 MEP synthase. The *Y. pestis* CO92 MEP synthase displays 50% identity (71% homology) to the *F. tularensis* MEP synthase, which we have previously characterized [1]. Through a series of enzyme assays, the relevant kinetic parameters for the *Y. pestis* enzyme were determined (apparent K_M^{DXP} = 252 μM, apparent K_M^{NADPH} = 12.7 μM, divalent cation specificity towards Mg^{+2} or Mn^{+2}).

Due to a low yield of purified protein obtained from a codon optimized *Y. pestis* MEP cytidylyltransferase expression construct, we elected to design PCR primers to amplify the native MEP cytidylyltransferase gene from *Y. pestis* CO92 genomic DNA, based upon our prior success with expression and purification of the native *F. tularensis* MEP cytidylyltransferase [2]. We obtained a PCR product of the expected size, cloned the product into a pET101D plasmid for protein expression in *E. coli* BL21(DE3) RIL Codon Plus, and confirmed the acquisition of the desired clone by restriction mapping. Unfortunately, protein expression and purification still resulted in very poor yields of recombinant protein. Furthermore, the purified protein was found to be devoid of any catalytic activity. Hence, we elected to focus our effort on the *F. tularensis* MEP cytidylyltransferase in lieu of the *Y. pestis* homolog.

Aim 2) Optimize HTS assay conditions for MEP synthase and MEP cytidylyltransferase.

Since purified recombinant *Y. pestis* MEP synthase was found to have significantly greater specific activity than the *F. tularensis* homolog, the *Y. pestis* enzyme appears better suited to large scale high-throughput screening (HTS) as less protein is needed per assay, thereby reducing the per-well cost of a screen. The quality and robustness of an enzyme assay are important considerations for the reliable screening of a molecular library, and are typically described in terms of the Z-factor [3]. Ideally, an assay should have a large dynamic range (the difference between the uninhibited and inhibited signals) and small standard deviation across replicates, which corresponds to a Z-factor score near a value of 1 (an assay with a Z-factor score between 0.5 and 1.0 is considered excellent for screening). To determine the Z-factor for the spectrophotometric assay using the *Y. pestis* MEP synthase, we fixed the DXP concentration to the K_M, used a saturating concentration of NADPH (150 mM), and evaluated three separate lots of purified enzyme in a series of assays performed over three consecutive days. FR900098 was used as a positive control for inhibition. The Z-factor was determined to be 0.9, indicative of an assay well suited for library screening. Additionally, the Z-factor for the *F. tularensis* MEP cytidylyltransferase assay was found to be 0.8 [2], also well suited to a HTS campaign.

Aim 3) Provide purified recombinant *F. tularensis* MEP synthase and MEP cytidylyltransferase for crystallization and structure determination.

We have provided the purified recombinant *F. tularensis* proteins to WRAIR in an on-demand basis.

Aim 4) Provide purified recombinant *Y. pestis* MEP synthase and MEP cytidylyltransferase for crystallization and structure determination.

We have provided the purified recombinant *Y. pestis* MEP synthase to WRAIR in an on-demand basis. Further optimization of *Y. pestis* MEP cytidylyltransferase expression is necessary.

Aim 5) Evaluate structure-activity relationships of rationally designed inhibitor molecules in enzyme-based assays.

As reported in the 2012-2013 progress report, to obtain an initial evaluation of the performance of the purified *Y. pestis* MEP synthase in a small scale high-throughput type of assay, we elected to screen our in-house, natural product, phytochemical library of molecules using bench scale assay volumes (120 µL). As illustrated in Figure 2, four inhibitor hits were obtained in the screen (demonstrating <25% residual enzyme activity).

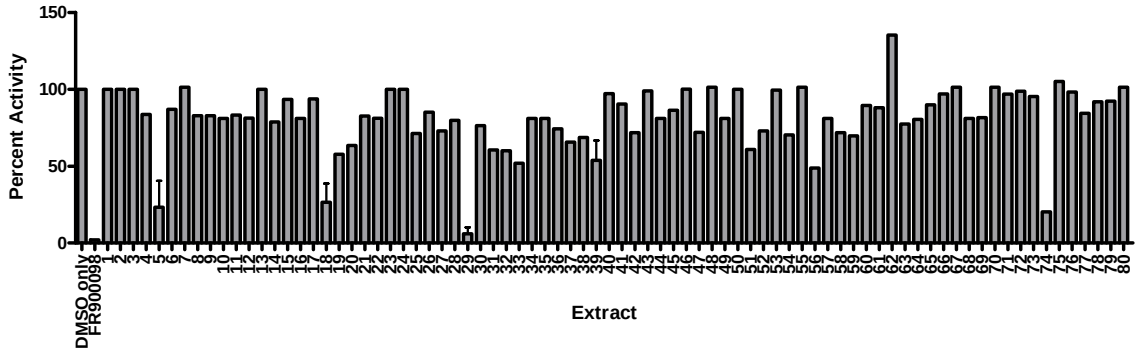


Figure 2. Library screening with purified *Y. pestis* MEP synthase. Enzyme activity, relative to uninhibited enzyme (DMSO only) is shown. The phytochemical library consists of natural product extracts from a variety of domestic plants. Hence, each extract contains multiple compounds.

Follow-on inhibition assays with the 4 top hits confirmed that the greatest activity is associated with extract 29 (Figure 3). A *Y. pestis* growth inhibition assay demonstrates the dose-dependent activity of e29 (Figure 4). We then performed detailed kinetic evaluation of extract 29 (Figure 5) and deduced the presence of a new class of MEP synthase inhibitor, functioning by binding to a previously unknown allosteric site on the enzyme (to date, all known inhibitors of MEP synthase are competitive, binding in the active site of the enzyme, while this new inhibitor binds to an allosteric site outside of the active site). We have recently confirmed the allosteric activity with purified recombinant *F. tularensis* and *Mycobacterium tuberculosis* MEP synthase. This exciting discovery affords the development of a completely new family of antibiotics targeting MEP synthase. Efforts to identify this novel inhibitor are currently underway.

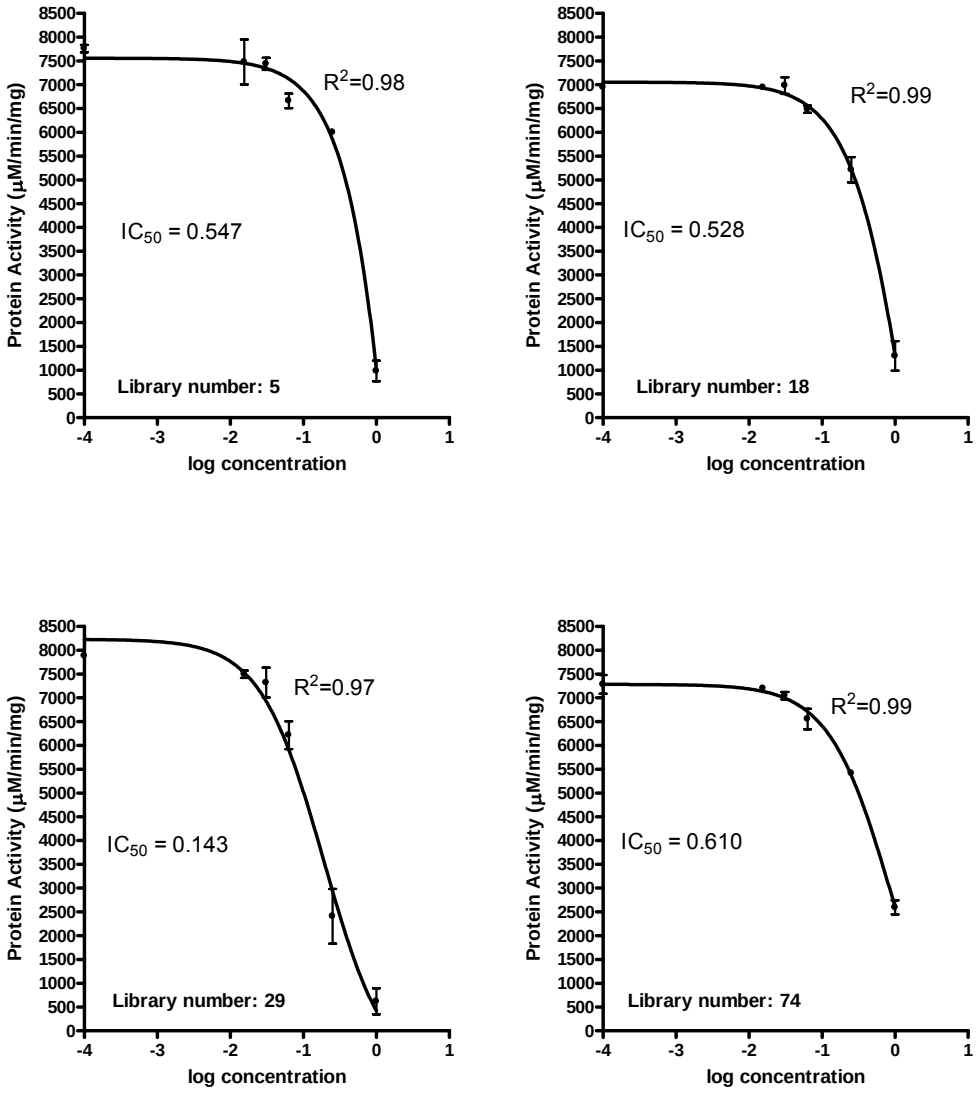


Figure 3. Dose-response plots of the top 4 hits identified in the library screening. Extract 29 demonstrates the greatest relative potency (note: since the extracts are mixtures of several molecules, the IC_{50} values are unitless).

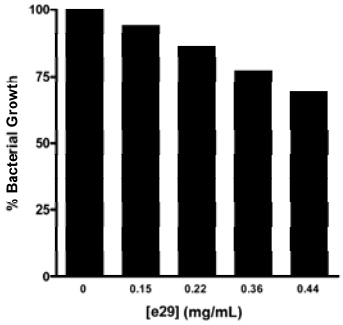


Figure 4. *Yersinia pestis* growth inhibition assay with extract 29. *Y. pestis* A1122 was cultured in the presence of e29 at the indicated concentrations. Bacterial growth is relative to an uninhibited culture. All assays were performed in duplicate. The extract inhibits bacterial growth in a dose-dependent fashion.

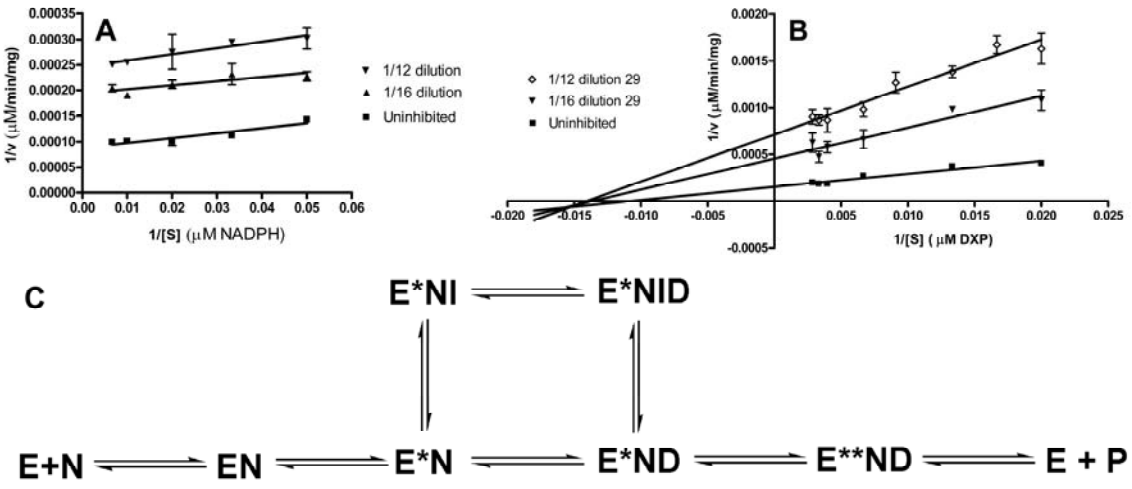


Figure 5. Mechanism of inhibition by e29. A) Relative to NADPH, e29 is an uncompetitive inhibitor of the purified *Y. pestis* MEP synthase. B) Relative to DXP, e29 is a noncompetitive inhibitor. C) A model of e29 inhibition. MEP synthase (E) undergoes a conformational change (E*) upon binding of NADPH (N), exposing an allosteric site to which the inhibitor (I) binds. As the inhibitor is noncompetitive with respect to DXP (D), I may bind the E*N or E*ND complex, thereby inhibiting the enzyme.

Rational Library Screening - MEP synthase

To identify additional lead compounds for the development of novel small molecule inhibitors of MEP synthase, we initiated a collaboration with Prof. Cynthia Dowd at George Washington University and screened two molecular libraries for inhibitory activity against the purified *Y. pestis* enzyme. The first library consists of 50 rationally designed synthetic compounds, primarily modeled on the structures of the *M. tuberculosis* MEP synthase in complex with fosmidomycin or FR900098 [4], [5]. As introduced elsewhere [6], the strategy for the synthesis of this library was to create novel compounds with either amide- or O-linked substituents appended to the retrohydroxamate moiety of fosmidomycin/FR900098, thereby targeting the two major binding sites in MEP synthase; the fosmidomycin/DXP site and the NADPH site, bridging these adjacent sites to yield a highly specific ligand. Select structures of the inhibitors are shown in Figure 6. As anticipated, when screening this rational library against purified *Y. pestis* MEP synthase, several of the compounds were found to demonstrate significant inhibitory activity (>75% inhibition), as illustrated in Figure 7. The top five inhibitors were subsequently evaluated in dose-response assays (Figure 8), with compounds **15** and **16** demonstrating the greatest potency. Due to the potential for competitive bisubstrate

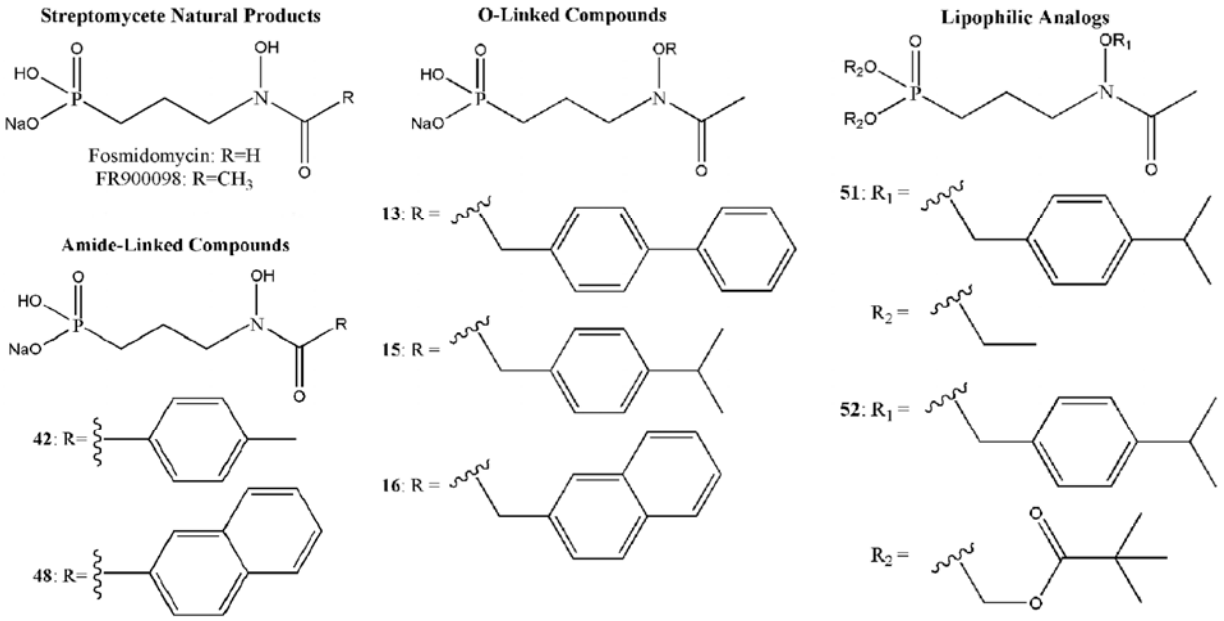


Figure 6. MEP synthase inhibitors. The structures of fosmidomycin, FR900098, and select rationally designed amide-linked and O-linked inhibitors are shown, including lipophilic prodrug analogs of compound **15**.

inhibition, we also evaluated **15** and **16** by preincubating the enzyme with inhibitor prior to the addition of NADPH or DXP (in contrast to the assays depicted in Figure 8, wherein the enzyme was concomitantly exposed to NADPH and the inhibitor). As shown in Figure 9, the resulting IC_{50} values for compounds **15** and **16** improve approximately 3- and 12-fold, respectively, supportive of competitive inhibition relative to both NADPH and DXP. It is particularly noteworthy that the IC_{50} for compound **16** (0.3345 μM) approximates one half of the MEP synthase concentration used in the assay, indicative of a tight-binding inhibitor.

To further explore if compound **16** inhibits by occupying both the DXP and NADPH binding sites, we next performed inhibitor modality assays with the purified *Y. pestis* MEP synthase. Catalysis by MEP synthase involves an ordered bi bi reaction mechanism, wherein NADPH must bind to the enzyme before DXP [7]. This mechanism is indicative of an underlying conformation change accompanying the binding of NADPH, thereby resulting in the formation of the DXP binding site. Accordingly, relative to DXP, fosmidomycin and FR900098 are competitive inhibitors of MEP synthase, while they are uncompetitive with respect to the binding of NADPH [7] [6] (Figure 10). Hence, NADPH must first bind to the enzyme before fosmidomycin/FR900098 can compete with DXP for its binding site.

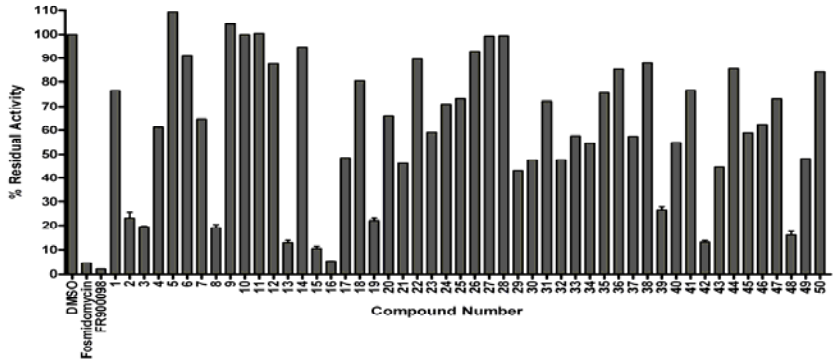


Figure 7. Screening a rationally designed molecular library. The *Y. pestis* MEP synthase was assayed in the presence of 100 μM of the indicated inhibitor. Residual activity is relative to the assay performed with vehicle alone (DMSO). All assays were performed in duplicate. Of those compounds tested, five inhibit the enzymatic activity by >75%, including compounds 13, 15, 16, 42, and 48.

In light of the fosmidomycin and FR900098 mechanism of inhibition, and given the anticipated mechanism for the bisubstrate inhibitor **16**, we performed mode of inhibition assays in each of two ways; the first with **16** added after preincubating the enzyme with NADPH (Figure 11) and the second with compound **16** preincubated with the enzyme prior to the addition of any other substrates (Figure 12). As illustrated in Figures 11 and 12, compound **16** is competitive with respect to DXP and competitive with respect to NADPH, under either of the two assay conditions. Thus, in contrast to fosmidomycin and FR900098, compound **16** does not require the initial binding of NADPH to the enzyme. In fact, as it competes with NADPH for a binding site, its activity is more potent when preincubated with MEP synthase in the absence of NADPH (contrast the concentrations of **16** used in the plots shown in Figures 11 and 12). Due to its ability to bind to the NADPH site, compound **16** appears capable of promoting the same structural change in the enzyme as does NADPH, causing the ensuing formation of the DXP binding site. Consequently, compound **16** behaves as a tightly bound inhibitor, binding to the NADPH site and causing a conformation change that subsequently "locks" the inhibitor into the DXP site. Further exploration of this mechanism is currently underway.

Secondary to the enzyme assays, compounds **15** and **16** were also evaluated in a growth inhibition assay with liquid cultures of *Y. pestis* A1122. As shown in Figure 13, at 500 μM, compounds **15** and **16** demonstrate inhibitory activity comparable to FR900098 and fosmidomycin. Additionally, two lipophilic esters of compound **15** (compounds **51** and **52**) also demonstrate effective growth inhibition. Hence, inhibitors **15** and **16** appear to be excellent lead molecules warranting further development.

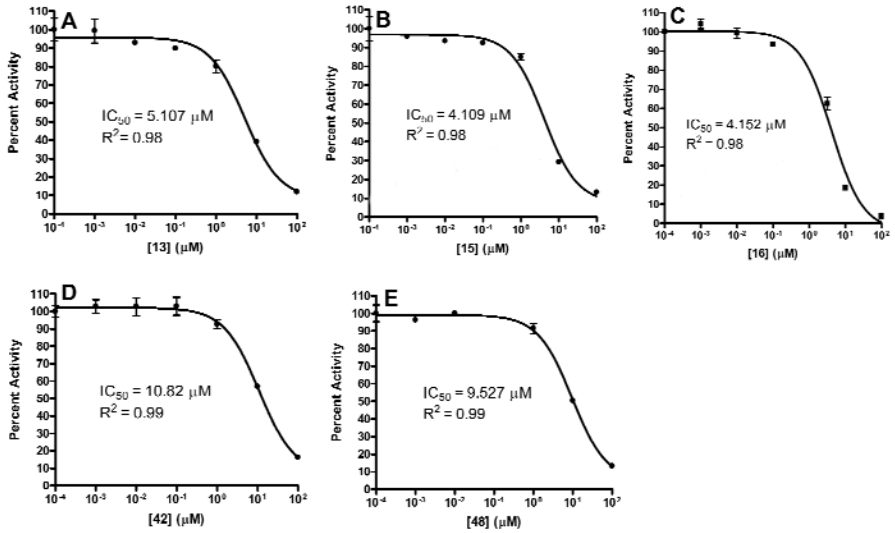


Figure 8. Dose-response plot of *Y. pestis* MEP synthase with the top five rationally designed inhibitors; compounds A) 13, B) 15, C) 16, D) 42 and E) 48. Assays were performed by combining the enzyme with 150 μM NADPH, followed by addition of the inhibitor. After five minute incubation at 37 ^{o}C, substrate was added to initiate the reaction. The R^2 value for each plot is indicated. The enzymatic activity is relative to an uninhibited control. All assays were performed in duplicate.

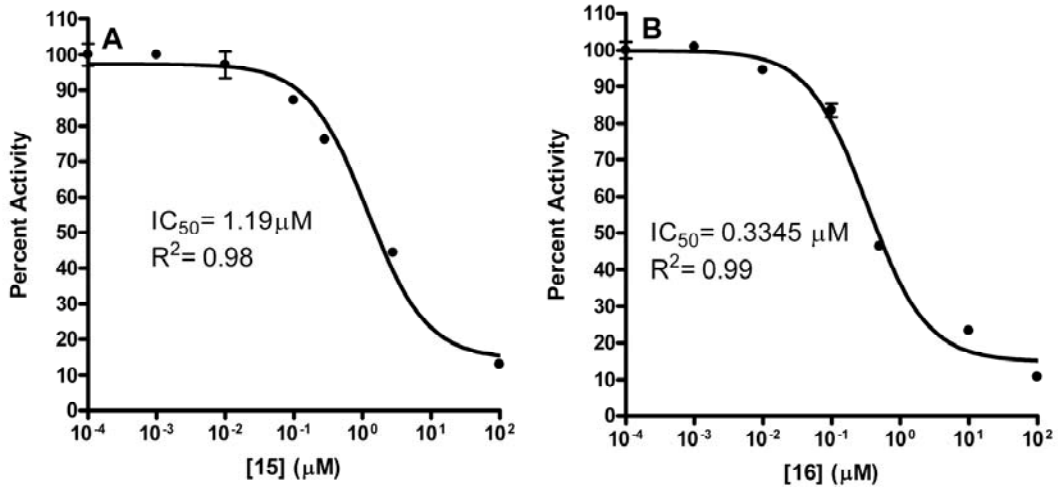


Figure 9. Dose-response plot of the *Y. pestis* MEP synthase when preincubated with the inhibitor. Assays were performed by combining the enzyme with either A) compound 15 or B) compound 16 and preincubating at 37 ^{o}C for 10 min before addition of NADPH and DXP. All assays were performed in duplicate. Activity of the enzyme is relative to an uninhibited control.

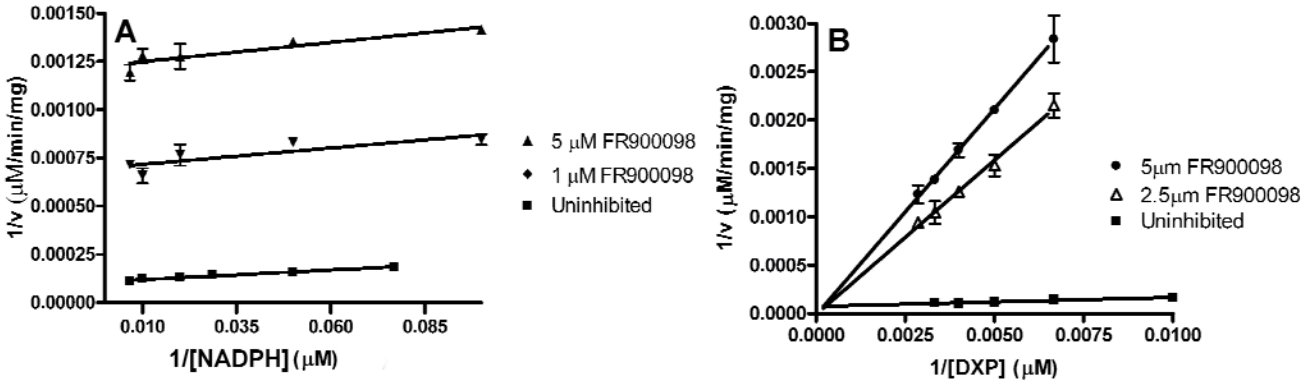


Figure 10. Mode of inhibition by FR900098. The Lineweaver–Burk plots indicate that FR900098 is uncompetitive with respect to NADPH (A), but competitive with respect to DXP (B). All assays were performed in duplicate using purified *Y. pestis* MEP synthase.

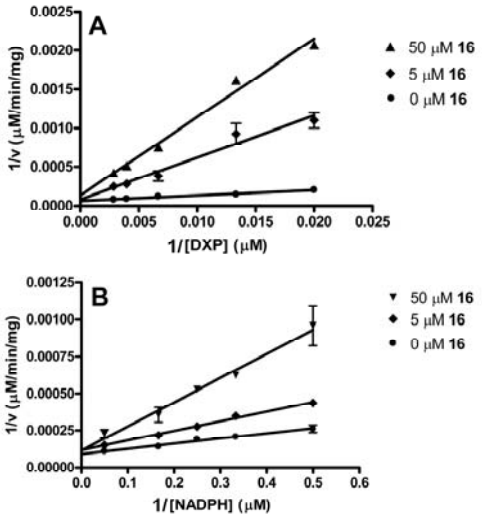


Figure 11. Mode of inhibition by compound **16**. The Lineweaver–Burk plots indicate that compound **16** is competitive with respect to DXP (A) and competitive with respect to NADPH (B). All assays were performed in duplicate using purified *Y. pestis* MEP synthase. The enzyme was not preincubated with compound **16**, in contrast to Figure 12.

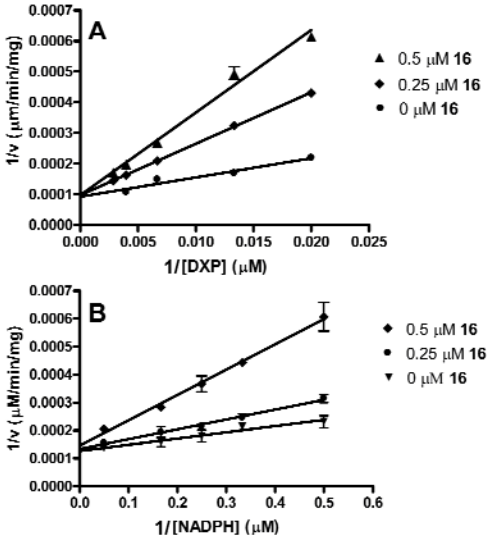


Figure 12. Mode of inhibition with preincubation. When the *Y. pestis* MEP synthase is preincubated with compound **16** (37 ^{o}C, 10 min) prior to the addition of NADPH and DXP, the Lineweaver–Burk plots still indicate that compound **16** is competitive with respect to DXP (A) and NADPH (B). All assays were performed in duplicate.

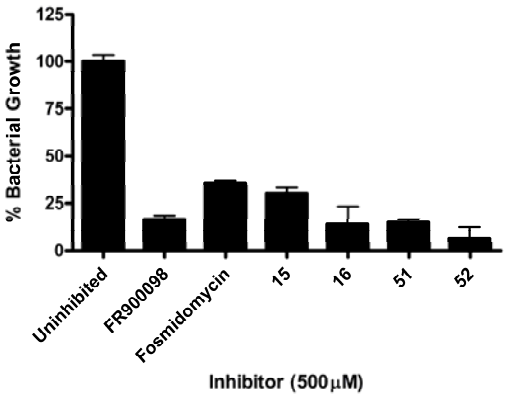


Figure 13. Growth inhibition assay with liquid cultures of *Y. pestis*. *Y. pestis* A1122 was cultured in the presence of 500 μM of the indicated inhibitor. Bacterial growth is relative to an uninhibited culture. All assays were performed in triplicate. Compounds **15**, **16**, **51**, and **52** have inhibitory activity comparable to fosmidomycin and FR900098.

Rational Library Screening - MEP Cytidylyltransferase

As presented in Figure 14, using purified *F. tularensis* MEP cytidylyltransferase, we have evaluated the inhibitory activity of 34 rationally designed inhibitors of MEP cytidylyltransferase (provided by WRAIR). While most compounds demonstrate only modest inhibitory activity, compounds 110039 and 401145 have significant activity (Figure 14). After determination of IC_{50} values with these two compounds and purified cytidylyltransferase (Figure 15), we ascertained the activity of the two compounds in bacterial growth inhibition assays. As shown in Figure 16, both compounds inhibit *Y. pestis* growth *in vitro*, although 110039 demonstrates significantly greater potency. A dose response plot with *Y. pestis* and 110039 indicates activity similar to that of FR900098 (Figure 17). Efforts are currently underway to determine the mechanism by which compound 110039 inhibits MEP cytidylyltransferase.

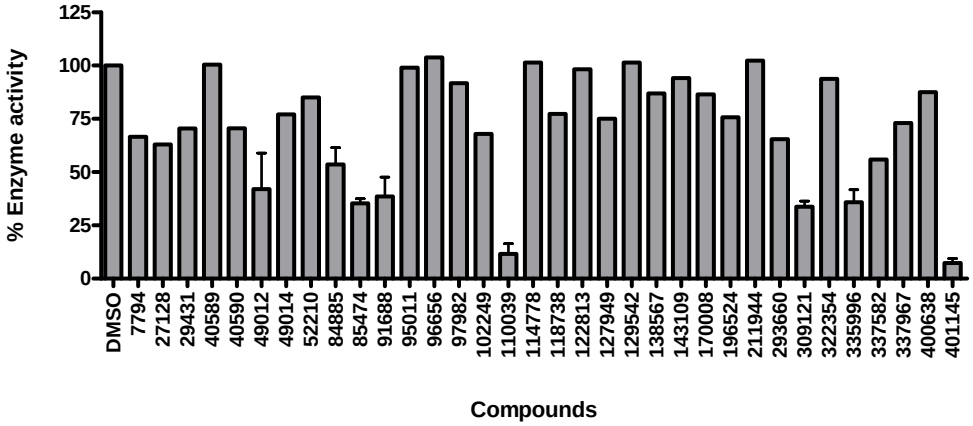


Figure 14. Screening a rationally designed molecular library. The *F. tularensis* MEP cytidylyltransferase was assayed in the presence of 100 μM of the indicated inhibitor. Residual activity is relative to the assay performed with vehicle alone (DMSO). All assays were performed in duplicate. Of those compounds tested, two inhibit the enzymatic activity by >75% (compounds 110039 and 401145).

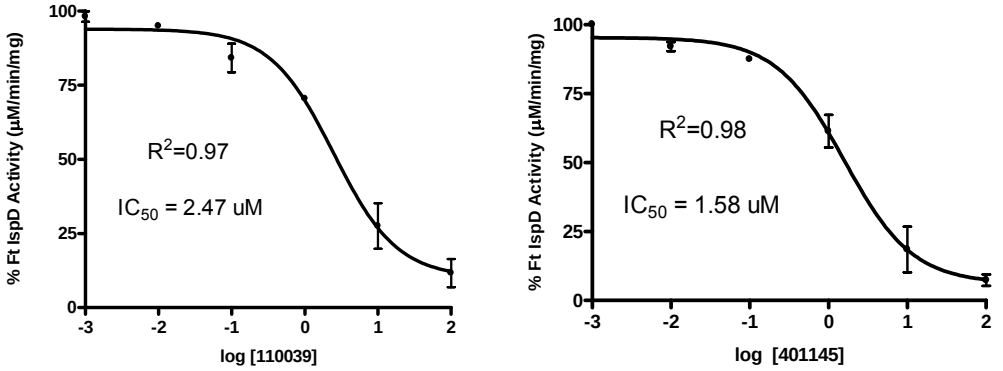


Figure 15. Dose-response plot of *F. tularensis* MEP cytidylyltransferase with the top two rational inhibitors; compounds 110039 (left) and 401145 (right). The R^2 value for each plot is indicated. The enzymatic activity is relative to an uninhibited control. All assays were performed in duplicate.

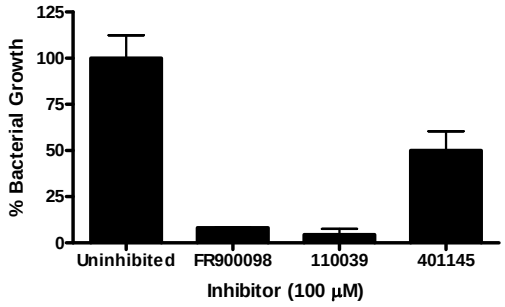


Figure 16. Growth inhibition assay with liquid cultures of *Y. pestis*. *Y. pestis* A1122 was cultured in the presence of 100 µM of the indicated inhibitor. Bacterial growth is relative to an uninhibited culture. All assays were performed in triplicate. Compound 110039 has inhibitory activity comparable to FR900098.

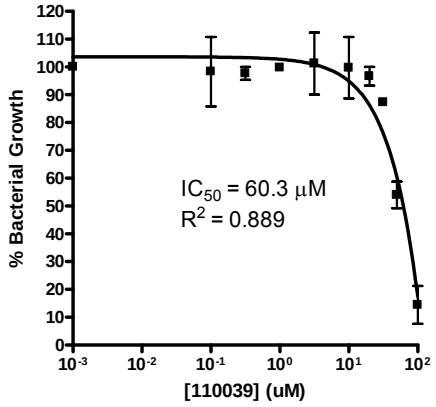


Figure 17. Dose-response plot of *Y. pestis* A1122 cultured in the presence of 110039. Bacterial growth is relative to an uninhibited culture. All assays were performed in triplicate. Compound 110039 has an IC_{50} similar to FR900098 (see Figure 1).

Key Research Accomplishments

- Optimization of MEP synthase and MEP cytidylyltransferase assays for HTS.
- Screening of rationally designed MEP synthase and MEP cytidylyltransferase inhibitor libraries with the resulting identification of several hit molecules.
- IC_{50} determination and growth inhibition secondary screens to validate hits and identify top compounds.
- Mode of inhibition studies to determine the mechanism of action for the top hit compounds.
- Identification of a previously unknown allosteric site on the MEP synthase enzyme from *F. tularensis*, *Y. pestis*, and *M. tuberculosis*.
- On-demand production and delivery of recombinant proteins to WRAIR for X-ray crystallography.

Reportable Outcomes

- A manuscript detailing the characterization of the *Y. pestis* MEP synthase, including the identification of the allosteric site, has been submitted to PLoS ONE and is currently under review.
- Manuscripts detailing the assessment of several rationally designed MEP synthase inhibitors have been published:

  Chofor, R., Risseeuw, M.D., Pouyez, J., Johny, C., Wouters, J., Dowd, C.S., Couch, R.D., Van Calenbergh, S. Synthetic Fosmidomycin Analogues with Altered Chelating Moieties Do Not Inhibit 1-Deoxy-D-xylulose 5-phosphate Reductoisomerase or *Plasmodium falciparum* Growth *In Vitro*. Molecules. 2014; 19(2):2571-2587.

  Jackson, E.R., San Jose, G., Brothers, R.C., Edelstein, E.K., Sheldon, Z., Haymond, A., Johny, C., Boshoff, H.I., Couch, R.D., and Dowd, C.S., The effect of chain length and unsaturation on Mtb Dxr inhibition and antitubercular killing activity of FR900098 analogs. Bioorganic & Medicinal Chemistry Letters, 2014 Jan 15;24(2):649-53.

  San Jose, G., Jackson, E.R., Uh, E., Johny, C., Haymond, A., Lundberg, L., Pinkham, C., Kehn-Hall, K., Boshoff, H.I., Couch, R.D., and Dowd, C.S., Design of Bisubstrate Inhibitors of 1-Deoxy-D-xylulose 5-Phosphate Reductoisomerase (Dxr) from *Mycobacterium tuberculosis* (Mtb). *Med. Chem. Comm.* 2013 4:1099-1104.

- Funds for this project are used to support a lab technician (Ms. Chinchu Johny) and a graduate student (Ms. Amanda Haymond).

Conclusion

In summary, during this second fiscal period, we have optimized assays with the *Y. pestis* MEP synthase and the *F. tularensis* MEP cytidylyltransferase for use in HTS. The screening of natural product and rationally designed libraries has identified a novel inhibitor that binds to an allosteric site on MEP synthase. We confirmed this allosteric activity with the MEP synthase homologs obtained from *F. tularensis* and *M. tuberculosis*. This allosteric site has not been previously identified and represents a new site for the rational design of a new chemical class of antimicrobial drugs targeting MEP synthase. Additionally, our screening has highlighted a rationally designed bisubstrate inhibitor of MEP synthase that behaves as a tightly bound inhibitor, binding to the NADPH site and causing a conformation change that subsequently "locks" the inhibitor into the DXP site. And our initial screening has also identified an effective inhibitor of MEP cytidylyltransferase.

During the next fiscal period, we will focus on screening large and chemically diverse molecular libraries for additional inhibitors of MEP synthase and MEP cytidylyltransferase.

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